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The Use of Vascular Endothelial Growth Factor in Ligament and Tendon Repair

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Introduction:

Many individuals in their lifetime will face an injury that will impair the movement of their musculoskeletal system. In the over 33 million musculoskeletal injuries reported per year in the U.S., 50% involve tendon and ligament injuries (Leong et al., 2020). Each year in the United States, approximately 17 million ligamentous injuries require medical treatment. These treatments have an estimated economic cost of over $40 billion (Leong et al., 2020).

In the human body, tendons and ligaments help aid the musculoskeletal system in locomotion. Tendons connect muscle to bone, while ligaments connect bone to bone (Kannus, 2000). These structures are made of connective tissue that are formed using specific cell type. Further, unlike skin and muscles that have constant access to oxygenated blood, ligaments and tendons are hypoxic, which means there is low oxygen in the area. These conditions create specific difficulties for the healing process. This process can take months and years due to a lack of vascularization. Most vascularized tendons and ligaments have some capacity to heal and form scar tissue, but some tendons and ligaments cannot heal on their own and need surgical intervention, including the anterior cruciate ligament (ACL) and ulnar collateral ligament (UCL).

Tendons and ligaments are essential tissues needed to move the body. When they are damaged movement is impaired and it decreases the quality of life for many individuals. When the tendon or ligament is torn, surgery is needed to fix it. The problem with this is that surgery does not always guarantee full recovery and the chances of re-injuring that tendon or ligament increase. For example, in anterior cruciate ligament reconstruction (ACLR), athletes who tried to return to sports at the same level of play had a 29.5% risk of suffering a second ACL injury within 24 months of surgery (Paterno et al., 2014). In one study, patients who had a torn tendon or ligament only regained 83% mass of the original tendon/ligament mass (Kaeding et al., 2015). This is not a high success rate considering that for the tendon and ligament to function at total capacity, it is missing 20% of its mass, including collagen mass, which is needed to

maintain the tendon and ligament strength to prevent repeat tears. Following an injury, the healing process begins.

Phases of Healing:

Tendons are made of type I collagen, a protein produced by tenoblasts and tenocytes (Kannus, 2000). Tenoblasts and tenocytes are cells found on collagen fibers that make up the fibril. Ligaments are very similar, except they are made of type III collagen. When ligaments and tendons tear or rupture, it can take a long time to heal due to the tendons and ligaments not being highly vascularized (Leong et al., 2020). This healing process is broken into three phases: inflammation, proliferation, and remodeling. These phases overlap but are distinct by the cytokines that are used. Cytokines are small proteins called growth factors used in cell signaling in the immune system. The inflammatory phase begins as soon as the injury occurs by forming a clot around the damaged fibrils to prevent bleeding. When this happens, fibroblasts which help with collagen formation are released, which causes platelets from the cytokine to be released to cause local inflammation (Leong et al., 2020). The growth factors that cause this inflammation are transforming growth factor-β (TGF-β), insulin-like growth factor-I (IGF-I), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) (Leong et al., 2020). This inflammation causes the immune system to send extrinsic inflammatory cells, which clear the tendon or ligament of any debris or infection. Two days following the injury, the proliferative phase begins.

The proliferative phase is characterized by the extracellular matrix of the tendon or ligament expanding and increasing cellularity (Leong et al., 2020). This phase starts when the fibroblasts found on the collagen of the tendon and ligaments increase the production of TGF-β and IGF-I in order to increase cellular mass. Once this happens, tenocytes and fibroblasts promote angiogenesis, forming new blood vessels in tissue and muscles. This is done by having the tenocytes and fibroblasts produce VEGF to provide the cells with nutrients to promote

collagen repair and synthesis (Leong et al., 2020). Angiogenesis is highly important in tendon and ligament repair because collagen synthesis needs the continuous availability of oxygen. This means that during this phase, the production of VEGF by tenocytes and fibroblasts is at its highest. This healing phase takes two weeks to produce the right materials needed for the tendon and ligament, along with the extracellular matrix.

The final phase is the remodeling phase. This phase begins when new collagen is deposited in order to form a new fibril, the base unit of the tendon (Liu et al., 2021). The remodeling phase overlaps with the proliferative phase when the extracellular matrix decreases but the fibrous membrane of the fibrils increases. When this happens, the tenocytes on the collagen fibers align to create tension on the ruptured tendon or ligament, collagen III production decreases, and collagen I production increases. Collagen I has more crosslinks and tensile strength and is more pliable for the fibrils to increase their mass (Leong et al., 2020). This continuous replacement of collagen III by collagen I can take a few months to years following an injury depending on the availability of nutrients and oxygen to aid collagen formation and replacement. This highlights the importance of growth factors released during angiogenesis in healing. The newly formed tissue produced in this phase does not possess the same ultrastructural properties as before the injury (Liu et al., 2021), meaning that the tendon or ligament is not as strong in the injury area prior to injury. In some instances, tendons and ligaments do not enter this phase of healing without extrinsic help from doctors. For example, the rotator cuff and intra-articular ligaments do not enter the remodeling phase without reconstructive surgery (Liu et al., 2021).

Tendon and Ligament Surgical Reconstruction

When tendons and ligaments tear or rupture, complete healing will not occur without surgical intervention. One ligament that requires reconstructive surgery is the ulnar collateral ligament (UCL). This ligament is found on the medial side of the elbow and connects the ulna to

the humerus. The UCL helps provide support in the elbow joint and prevents angulation of the joint (Erickson at el., 2015). As a result of the joint being in the elbow, there is low vascularization, so the reconstruction phase cannot fully heal the ligament. Doctors have discovered that in order to prevent medial elbow pain after a rupture or return to a total joint motion by throwing overhand, reconstruction is needed. These UCL ruptures are often caused by repeated throwing motions as a result of the stress placed on the UCL during the cocking phase of a pitch motion (Erickson at el., 2015). The UCL reconstruction utilizes grafts anchored into the bone to replace the ruptured tendon. In surgical reconstruction, there are two types of grafts, an allograft and an autograft. An allograft is when tissue is transplanted from an outside source, whether that be from a cadaver or from synthetically made tissue (Kennon et al., 2020). An autograft is a graft of tissue from the same individual's body (Kennon et al., 2020). For a torn UCL, the graft options include the ipsilateral palmaris longus tendon autograft, gracilis or semitendinosus autograft, and an allograft (Erickson at el., 2015). The ipsilateral palmaris longus tendon is found in the anterior forearm, interposed between the Flexor Carpi Ulnaris and the Flexor Carpi Radialis muscles (Figure 1) (Pal et al., 2016).

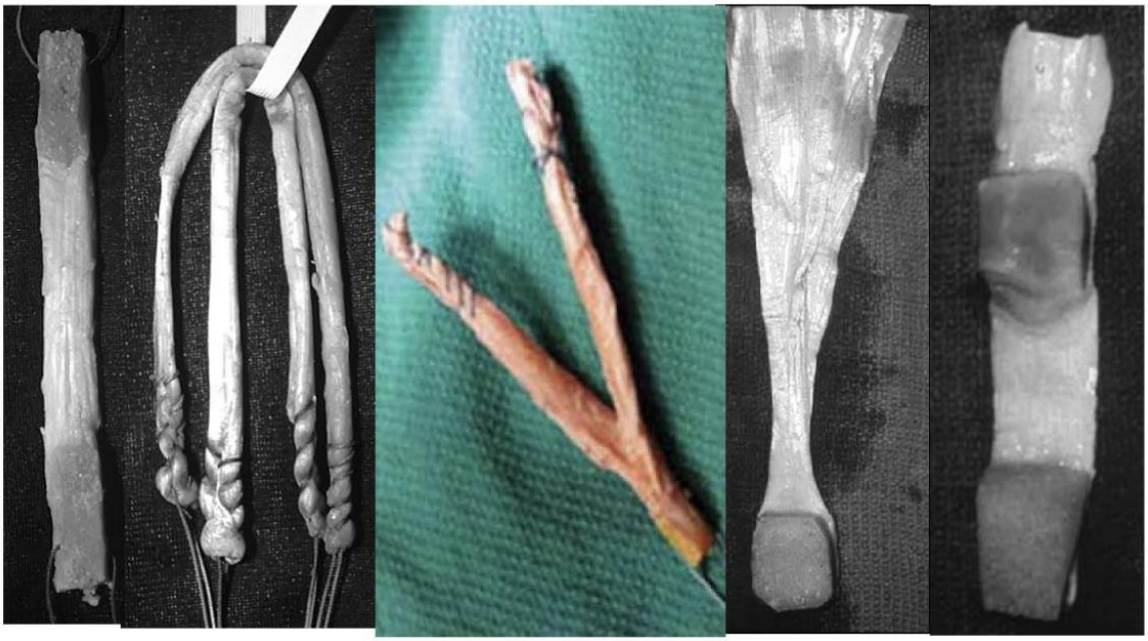

Figure 1: From Left to Right: Bone-Patellar Tendon Bone (BTB) Autograft, Quadrupled Hamstring Autograft, Split Quadriceps Tendon Autograft with bone block, Achilles Tendon Allograft, Whole Patellar tendon Allograft (Mall, MD, 2012)

Before the allograft can be inserted, it is first harvested from the forearm and is then anchored to the humerus and ulna after anchor holes are drilled. Once inserted, the bone around the ligament heals, creating a new UCL. Gracilis and semitendinosus autografts have a very similar process. The gracilis is harvested from the medial compartment of the thigh, and the semitendinosus is harvested from the posterior medial thigh. These are strong autografts due to them being made of muscle tissue instead of connective tissue like a tendon or ligament (Pal et al., 2016). The muscle will not weaken over time due to the graft having access to the same nutrients in the body as it had before. In UCL reconstruction, the tissue is transplanted the same way as an autograft, but unlike the autograft, the tissue is not from the patient's body.

In tendon reconstruction, the process is very similar to ligament reconstruction, but tendon reconstruction is more difficult. This is because tendon reconstruction often lacks sufficient donor tendon materials for grafting, along with having smaller tendon anchorages following surgery (Chattopadhyay at el., 2015). The grafts used during these reconstruction surgeries are palmaris, plantaris longus, and toe extensor tendon autografts. These autografts

often pose a problem when reconstruction is needed for the Achilles tendon or any other tendon reconstruction not in the hand or foot because the tissue required to repair the tendon often exceeds what is given from the autograft (Chattopadhyay at el., 2015). This means that in most reconstructions, the allograft is used from a cadaver or from synthetics. Another problem faced with tendon reconstructions is the anchorage sites. When tendon reconstructions are done in the hands or feet, the anchorage sites cannot be deep out of fear of going through the bone. This shallow anchorage may cause the newly reconstructed tendon to rupture again by falling out of the anchorage. As a result of this, tendon reconstructions use both tendon anchorage and the use of Fibrin glue to hold the tendon in place while healing (Soreide et al., 2018). The shortcomings of these surgical strategies highlights the need for new approaches for patients.

To prevent pain and decrease swelling in the area of a tendon or ligament injury, doctors prescribe corticosteroid injections. Corticosteroid mimics the body's hormonal response to decrease inflammation and reduce pain. These injections are put into the joint exogenously (around the cells of the tendon or ligament). Unfortunately, instead of helping the healing process, this course of treatment harms it. Due to the corticosteroids reducing inflammation, it inhibits the healing process as inflammation is needed to form clots and release the needed growth factors. This inhibition results in a sub-optimal outcome for healing (Speed, 2001). Recently, doctors and surgeons have been looking into a way to increase healing without harming the outcome of tendon and ligament reformation. A course of treatment that has come into development is platelet-rich plasma (PRP) injections. This is when plasma (cells from the blood that contribute to blood volume) and platelets (blood cells with the role of healing) are taken from the patient and made into an injection. This solution is then injected into the joint of the patient to increase healing. Doctors have discovered that if PRP injections are combined with growth factors, healing can increase. One growth factor, in particular, that increases blood flow to the area of tendons and ligaments is the vascular endothelial growth factor (VEGF).

History of VEGF

In the mid-1900s, intravascular disease was found to be the leading cause of vision loss. It was hypothesized that the disease caused the retina to produce an abnormal vascular growth called Factor X (Miller J, 2016). Later in 1971, Judah Folkman hypothesized that tumor growth was reliant on angiogenesis. In a study that he conducted, he found that tumors did not grow more than a millimeter without producing capillaries and blood vessels. With this, he believed that tumors had to secrete a substance that would stimulate cells to increase capillary formation (Cao, 2008). He also believed that if this factor was discovered, it could be used as a therapeutic target for cancer treatment. Then finally, in 1989, the first to isolate and clone vascular endothelial growth factor (VEGF) was Napoleone Ferrara. In 1993 he found that inhibition of VEGF-induced angiogenesis by using antibodies resulted in the suppression of tumor growth (Ferrara N, 2011). With this discovery, Ferrara helped develop Avastin, a humanized anti-VEGF monoclonal antibody, in 1997 that passed the clinical trial and is now used to treat tumors in millions of patients (Ferrara N, 2011).

With these findings and VEGF's relation to angiogenesis, scientists began to look at its role in the inflammation process in tissues. Scientists knew that VEGF played a role in tissue healing due to the tissue being highly vascularized and the need to perform angiogenesis. When blood flow is restored to tissue, oxygen, and nutrients are returned and help support the growth and function of reparative cells (Johnson et al., 2014). Studies have found that the higher the amount of VEGF found in the damaged tissue, the more vascularization (Johnson et al., 2014). It was first believed that the VEGF could only be found in the lining of endothelial cells. Now scientists know that VEGF can be expressed by a variety of other cell types involved in healing. With this discovery, scientists began to experiment with how this growth factor could affect different cells found in the body. A demand for ligament and tendon healing treatment led scientists to consider the use of VEGF (Johnson et al., 2014).

VEGF in Healing

VEGF is an angiogenic factor that regulates blood vessel formation in tendon and ligament healing (Liu et al., 2021). VEGF is most active just after a tendon or ligament injury and continues to influence the functions of various processes (Molloy et al., 2003). VEGF is secreted by platelets at the site of injury and promotes angiogenesis in the proliferative phase of healing. During angiogenesis, VEGF promotes vascular wall permeability and the growth of vascular endothelial cells and perivascular cells (Liu et al., 2021).

Further, VEGF also promotes fibroblast proliferation and the production of other growth factors. VEGF has seven sub-proteins that are used in angiogenesis, these are VEGF-A, VEGF-B, VEGF-C, VEGF-D, Placental Growth Factor (PlGF), virus-encoded VEGF-E, and the snake venom-derived VEGF-F (Liu at el., 2021). VEGF-A is the most potent stimulator out of these six sub-proteins, meaning it is found in the greatest abundance during angiogenesis. VEGF production during angiogenesis is endogenous and exogenous, meaning inside and outside the site of healing. Figure 2 expresses the growth factors produced by platelets.

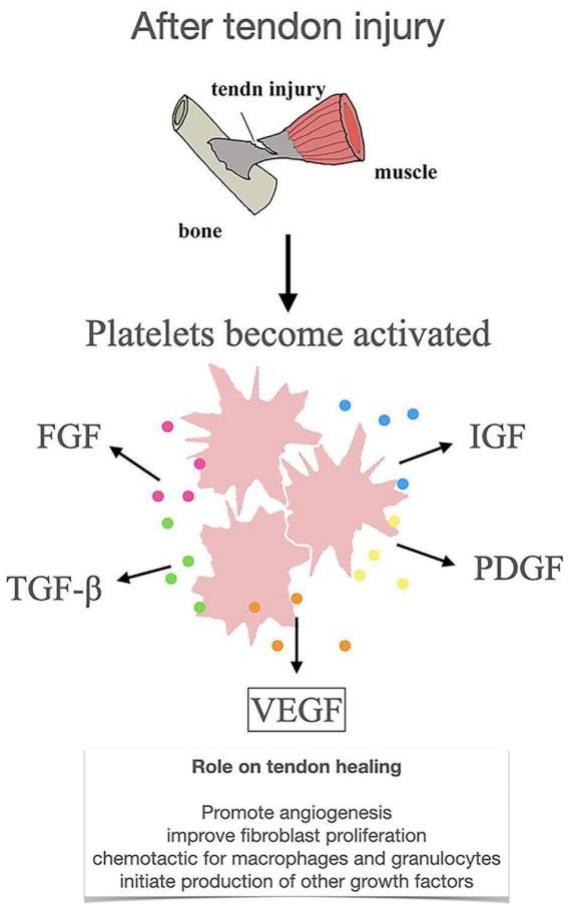

Figure 2: The process of VEGF expression in platelet cells before angiogenesis. (Liu at el., 2021)

In a healthy tendon or ligament, VEGF production is negligible. After a tendon or ligament injury, VEGF levels rise. In a study of a dog's synovial flexor tendon, VEGF production peaked on day seven after injury and then decreased on day 21 (Liu at el., 2021). This demonstrates that VEGF expression is at its highest during the early stage of proliferation and then decreases after. Further, VEGF expression changes are based on if an injury is chronic or acute. In order to increase VEGF expression, there are four factors: hypoxia, inflammatory cytokines, nerve signals, and mechanical load (Pufe et al., 2005). This is because, after a tendon or ligament injury, the hypoxic condition causes the expression of hypoxia-inducible factor-1 (HIF-1), which induces transcription of the *vegf* gene. VEGF synthesis increases when inflammatory cytokines are released. Next, after an injury to a tendon or ligament, neural fibers

increase due to the expression of nerve growth factor (NGF). This expression then causes VEGF production to increase in order to support neural fiber growth. Finally, when a tendon or ligament is overused, it causes tendinopathy, which means the tendon or ligament becomes inflamed. As a result of this, it causes the mechanical load to increase, which causes the expression of VEGF to increase (Liu et al., 2021).

VEGF is a mediator of angiogenesis. The most prominent form of VEGF found in the body is VEGF-A. VEGF-A secretion is induced by ischemia and inflammatory stimuli. VEGF-A is transcribed when the local cellular environment is changed, for example, if the cellular environment changes from hyperoxic to hypoxic. Once secreted, cellular responses to VEGF-A are mainly driven by their binding to their cognate receptor—the vascular endothelial growth factor receptors (VEGFRs) (Peach et al. 2018). VEGFRs are type I membrane proteins that contain an extracellular ligand-binding domain. This means that the domain for binding is found on the outside of the cell membrane. Once a ligand is bound, the VEGFR transmits information through a transmembrane domain to the cytoplasm of the cell (Leppänen at el. 2010). VEGF-A can bind to both VEGFR1 and VEGFR2. In mammals, VEGF-A signaling through VEGFR-2 is the major angiogenic signaling pathway (Brozzo et al. 2012). Binding to VEGFRs leads to endothelial cell proliferation, survival, migration, and vascular permeability. VEGF-A is produced by the transcription of the *vegfa* gene found in humans on chromosome 6p21 at exon 8 (Peach et al. 2018). Alternative splicing of the *vegfa* gene can lead to different isoforms of VEGF-A, which can alter signaling outcomes by affecting the binding affinity of VEGF-A and VEGFR2 (Peach et al. 2018).

The mechanism by which VEGFRs are activated is not fully understood, though it is predicted that the mechanism is similar to RTK activation. To activate RTKs, ligand-mediated dimerization with precise positioning of receptor subunits in active dimers is required (Brozzo et al. 2012). In the case of VEGF-A, VEGF-A is the ligand that binds to the RTK, which causes

dimerization. Active VEGFRs cause cell signaling to begin and promote endothelial cell migration and proliferation, as well as vessel formation (Brozzo et al. 2012).

Angiogenesis, the growth of new blood vessels from existing vessels, is an important aspect of the repair process (Johnson et al. 2014). This is because an increase in blood flow to damaged tissues provides oxygen and nutrients to support the growth and function of reparative cells. High levels of VEGF are found in normal wound and tissue repair, which results in a vigorous angiogenic response. Insufficient wound vascularization due to low VEGF activity contributes to delays in the repair process (Johnson et al. 2014). The importance of VEGF in the repair process is as a proangiogenic factor. Animal studies have shown that augmenting VEGF results in accelerated healing (Johnson et al.2014). Also, animals that have reduced levels of VEGF tend to heal more slowly. Human studies have suggested that low VEGF activity contributes to chronic, nonhealing wounds and tissue (Johnson et al. 2014).

In 2014, research was done on how much VEGF was produced by fibroblasts under normal conditions in tendons. The study found that a regular tendon fibroblast produces a small amount of VEGF. The study then focused on the amount produced after a tendon injury. It was found that VEGF production increases after an injury (Kaux et al., 2014). Current research is beginning to see how VEGF injected into the tendons and ligaments of animals affects the healing process. In 2021, the first human injection of VEGF post-surgery was done on a reconstructed Achilles tendon (Morimoto et al., 2021). More research must be done before further human clinical trials can be performed, and it is an ever-evolving treatment for tendon and ligament injuries. The literature review will focus on the outcomes of human and animal case studies and how VEGF affects tendon and ligamental healing when injected endogenously and exogenously. This is important for the medical field because surgeons need to implement better treatments that will improve patient healing and return patients to normal activities following treatment.

Endogenous VEGF Treatment:

Endogenous treatment involves injecting the VEGF growth factor directly into the tendon or ligament. This means that the injection must penetrate the connective tissue with the use of a needle and syringe. In most cases, the needle is guided via ultrasound inside the joint to ensure the contents of the syringe are delivered in the tendon. In a study done by Kaux et al (2014), VEGF-111 was injected into the Achilles tendon of rats. This study aimed to evaluate whether VEGF-111 could be used as a therapeutic treatment in tendon pathologies. VEGF-111 is a biologically active proteolysis-resistant VEGF-A splice variant (Kaux 2014). This splicing gives VEGF-A a longer half-life, meaning it will function for longer in the tendon. The control group received a saline injection into the tendon, while the experimental group was injected with VEGF-111. After 5, 15, and 30 days, the Achilles tendons of 10 rats of both groups were sampled and submitted to a biomechanical tensile test, along with collagen III, tenomodulin (TNMD), and metalloproteinase (MMP-9) mRNA quantification of tendon tissue. At 5, 15, or 30 days post-surgery, 10 rats of both groups were weighed and euthanized so that the Achilles tendon could be harvested. Once harvested a traction test was completed to determine the force at rupture or ultimate tensile strength (UTS) of the tendon. The study found that five days after surgery the UTS was low in both the control and VEGF-111 groups. On days 15 and 30, the UTS was significantly higher in the VEGF-111 group (Figure 3)

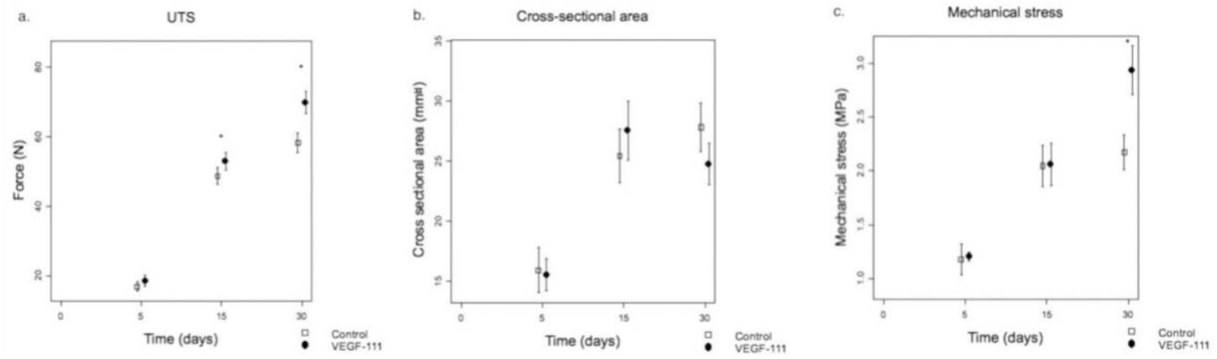

Figure 3: (a) Breaking strength of control and VEGF-111 groups at 5, 15, and 30 days. (b) Cross-sectional area of the tendon with time. (c) Calculated ratio between UTS and surface area of tendon and three points (Kaux 2014)

Next, the mRNA expression of collogen III (Col III), tenomodulin (TNMD), and matrix metalloproteinase (MMP-9) was quantified. It was found that Col III mRNA was highly expressed during the first 2 weeks and then decreased at day 30 in both the control and experimental groups. Then, the expression of TNMD was low on day 5, tended to increase on day 15, and decreased slowly on day 30. The expression of mRNA for the MMP-9 was stable in both groups at the three-time points but decreased in the VEGF-111 group after day 15.

These data show that VEGF-111 injection significantly improved the UTS of the healing Achilles tendons 15 and 30 days after surgery when compared to the control group. When looking at the mRNA data, there was a decrease in expression of MMP-9 mRNA at day 30. MMP-9 is a marker of tissue remodeling. This observation could be a result of decreased intratendinous inflammation, which could indicate advanced healing or a quicker healing process, and might support the improvement of the UTS of the VEGF-111 treated tendons. In conclusion, this preliminary experimentation showed that endogenous injection of VEGF-111 stimulated the tendon healing process by increasing the ultimate force of the tendons during healing in comparison with the control group (Kaux 2014).

In a study done by Tang et al. (2016), VEGF gene therapy was used via adeno-associated viral type-2 (AAV2) vector to overexpress the amount of VEGF intrinsically in the tendon of chickens. The VEGF gene therapy was introduced into a ruptured tendon via AAV2 vector to increase the expression of VEGF intrinsically in the tendon. By doing this, the study corrected the insufficiency of the tendon healing capacity by increasing VEGF expression. In the study, severed chicken flexor tendons were used and either AAV2-VEGF or sham AAV2 vectors (control) were injected into the tendon through micro-injection to both tendon stumps through cross-sections of the tendon cut (Tang 2016). The tendons were harvested over a 16-week period at 8-time points (weeks 1, 2, 3, 4, 6, 8, 12, and 16) (Tang 2016). These time points allowed for the data to cover the early, middle, and late tendon healing stages. qPCR and western blot were used to measure the expression of transferred VEGF genes in each of the groups. This allowed the experimenters to assess the changes in the expression levels of the *vegf* gene from post-surgical weeks 1 to 16. Western blots were done to quantify the amount of VEGF protein produced in the tendon. The VEGF transgene expression was the greatest in week 4, 2, and 3. The expression of the endogenous VEGF in the tendon treated with AAV2-VEGF had significantly increased when compared with the sham vectors or non-injection control. The takeaway from this data is that VEGF gene delivery increases VEGF gene expression in healing tendons (Tang 2016). The next part of the study determined tendon strength by determining the amount of collagen type I and III. In tendon healing, there should be to increase in type I collagen and a decrease in type III collagen. Western blot analysis showed an increase in the expression of type I collagen in the AAV2-VEGF treated tendons. Further, there was a significant increase in type I at weeks 3, 4, 6, and 8 in AAV2-VEGF-treated tendons. Further, in the treated group, type III collagen gene expression was down-regulated in the early weeks after surgery, while in non-treated groups type I and III collagen genes were very low.

The study further tested the tensile strength of the tendons. It was found that VEGF gene delivery enhances the healing strength in the critical healing period (Tang 2016). It was found that from weeks 1 to 4, the non-injection or sham vector control tendons exhibited “no gain” in strength. It was found that there were earlier increases in strength after AAV2-VEGF treatment. The AAV2-VEGF injection increased the strength of the tendons by 68-91% starting at week 3 and up to week 8. There was also a greater increase in strength in the AAV2-VEGF treated tendon by 82–210% (Tang 2016). Finally, it was found that the overall rupture rate of repaired tendons was significantly greater in both control groups than in treatment groups (Figure 4). In this study, it was found that using VEGF genes through AAV2 vectors improved the tendon strength in the early and middle healing stages. Further, this therapy offers a way of improving tendon strength by producing an increase in strength by 68 to 210%, which is likely ample to prevent tendon gapping or disunion of the tendons (Tang 2016).

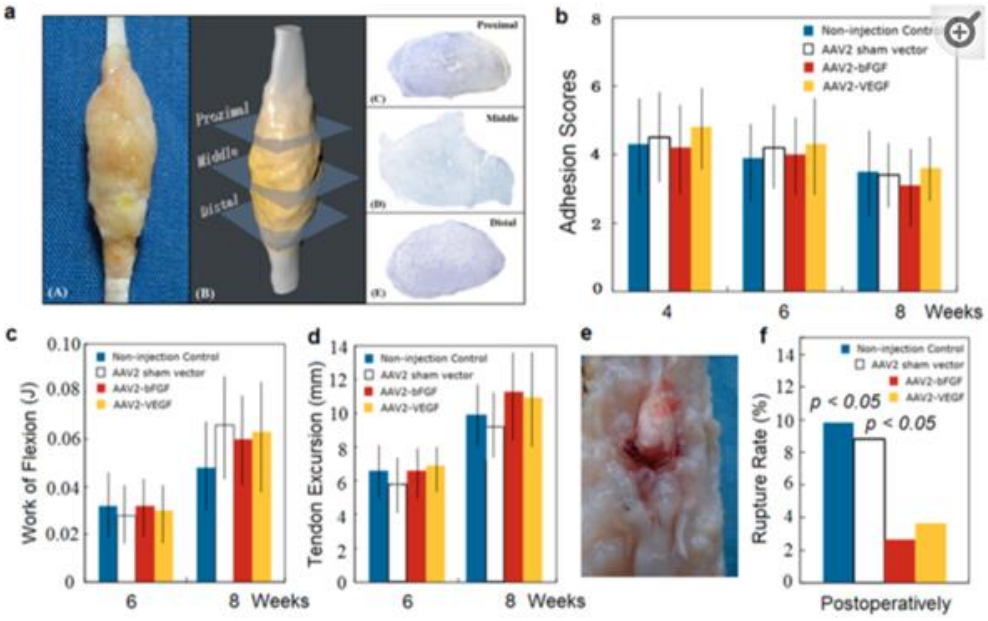

Figure 4: (a) 3D analysis method for quantification of adhesions around the tendon. (b) adhesion scores of each group. (c) Work of flexion of tendons in toes of treated and non-treated groups. (d) Tendon excursions under 10 N

load to the repaired tendon. (e) Picture of tendon rupture. (f) The overall rate of tendon ruptures during dissection in the samples for a mechanical test at weeks 4, 5, 6, and 8 (Tang 2016)

In a study done by Fan et al. (2016) it was found that VEGF promotes tendon regeneration in aged rats (6 months and older) by inhibiting the differentiation of tendon stem and progenitor cells and promoting vascularization. Recently, stem cells have been found in tendons called tendon-derived stem/progenitor cells (TSPCs), which have genes for tendon scleraxis (SCX) and tenomodulin (Tnmd) (Fan 2016). These cells play an important role in tendon maintenance and regeneration after injury. The study focused on cytokine expression during tendon repair of young and old rats by cytokine microarrays. The study hypothesized that cytokine VEGF is responsible for the accumulation of adipocytes (cells in connective tissue) in tendon healing. The study had two parts, the first looked at cytokine expression of patellar tendon repair in old and young rats. The second part looked at VEGF in aged rats.

For the first part of the study, the patellar tendon was ruptured by making a longitudinal cut in the middle of the patellar tendon of the rat. Then the tendon was removed at 3,7, and 14 days postoperatively to detect the expression of cytokines (Figure 5). For the second part of the experiment, the patellar tendon was ruptured, and cytokine VEGF was injected into the tendon shaft. The tendon was removed at 3,7, and 14 days postoperatively to detect the expression of cytokines. The study found that the cytokines IL-10, VEGF, and G-CSF showed a difference between aged and young rat tendons in the early stages. The expression of VEGF and G-CSF in the young group was highest on the third day. The study then found that VEGF enhanced tendon healing histologically and biomechanically in aged tendons. This was done by comparing the effects of tendon healing with and without VEGF injections using a scoring system. The system used Alcian blue staining to determine extracellular matrix organization, cell distribution, and organization to repair tissue. Using this scale, it was found that the control group at week four was 6.5±1.765 and the VEGF group was 8.25±1.765 (Figure 5B). The score of the VEGF

group was higher, meaning that the repaired tissue was more organized and the transition from repaired to healthy tissue was cleaner (Figure 5D). Collagen synthesis was determined with immunofluorescent staining of collagen types I and III. It was found that VEGF-treated groups had both collagen I and III and had a higher tensile strength than control groups. Further, VEGF was shown to increase vascularization by using an immunofluorescence staining experiment to determine CD31 and Von Willebrand factor (VWF). It was found that CD31 and VWF expression was highest in the VEGF injection group. CD31 and VWF are hormonal precursors for vascularization, and an increase in their expression indicates vascularization. In conclusion, the study found that VEGF expression increased in young tendons but decreased in old tendons. When VEGF was introduced to old tendons, it improved visualization and tensile strength, which indicated VEGF may improve the treatment of tendon diseases and injuries.

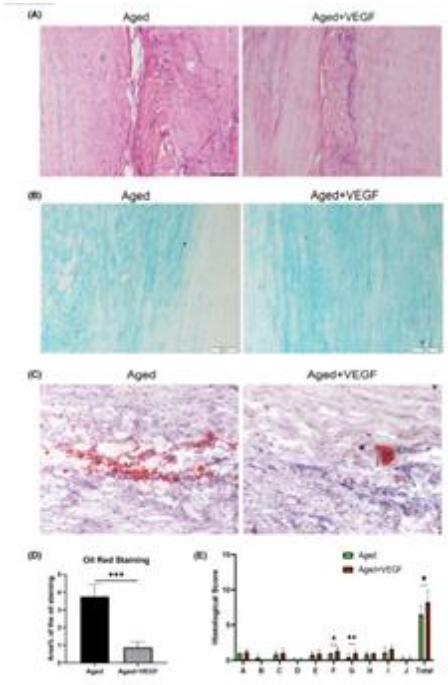

Figure 5: Histological analysis of aged tendons with and without VEGF treatment. (A) staining of aged tendons (B) aged + VEGF. (B) staining with alcian blue of aged and tagged+VEGF. (C) Adipocyte accumulation was reduced in aged+VEGF than just aged (amount of red dye). (D) Quantitive analysis of oil staining. (E) The histological score of the aged+VEGF group was higher than just the aged (Fan et al. (2016)).

In a human case study done by Morimoto et al (2021), an acute Achilles tendon rupture was treated with a combination of an intra-tissue injection of freeze-dried platelet-derived factor concentrate with VEGF. A 23-year-old male basketball player who had sustained an acute Achilles tendon rupture during a game was studied. The surgery was performed 4 days after the injury occurred. Four weeks postoperatively, freeze-dried platelet-derived factor (FD-PFC) concentrate was injected into the ruptured site of the Achilles tendon under ultrasound guide (Morimoto 2021). The FD-PFC that was injected into the site contained various growth factors to promote tissue repair and accelerate the healing process in various conditions. One of these factors was VEGF (Morimoto 2021). After injection, postoperative rehabilitation was performed. To see the results, an MRI was taken prior to surgery and 12 weeks after surgery (Figure 6). The postop MRI indicates healed rupture due to there being less white intensity in the heel compared to the preop scan. The case study found that by treating an Achilles tendon rupture with a combination of intra-tissue injection of FD-PFC and an early rehabilitation protocol after operative treatment, the patient could return to playing basketball at the pre-injury activity level only 3 months after the injury, compared to previous research showing that a rupture can take up to twelve months to return (Morimoto 2021).

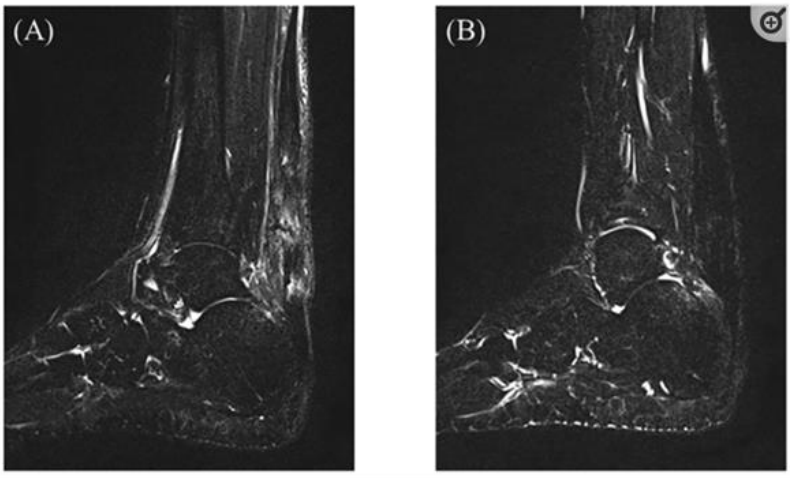

Figure 6: MRI imagining pre-operatively (A) and 12 weeks postoperatively (B). An increase in white intensity indicates high vascularized tissue (Morimoto et al. (2021)).

Experiments using Exogenous VEGF Treatment:

Exogenous treatment involves injecting the VEGF growth factor around a tendon or ligament. This means that the injection does not penetrate the connective tissue but is injected into the synovial fluid surrounding the tendon or ligament. Like endogenous treatment, a needle is guided via ultrasound inside the joint and the contents are released into the synovial fluid.

In a study by Setiawati et al. (2017), BM-MSCs and VEGF were injected into the distal tunnel at the articular site of rabbits after anterior cruciate ligament (ACL) reconstruction. The researchers aimed to determine how to stimulate graft and bone healing to gain an early return to daily activities, functional exercises, and sports. Bone marrow mesenchymal stem cells (BM-MSCs) were used, as they are an important source of cells for tissue repair. VEGF was tested due to it promoting angiogenesis, increasing capillary permeability, and contributing to fibrous integration between tendon and bone during the early postoperative stage. Four groups were tested: two controls with no injection (one group evaluated at 3 weeks, the other at 6 week, and two groups that received intratunnel injections of VEGF BM-MSC evaluated at 3 weeks and 6 weeks). The study found that collagen III expression was significantly higher in the treated group than in the control group at both 3 weeks and 6 weeks (Figure 7). This indicates that the ligament is healing with the treatment, as collagen III is the first collagen deposited to form fibrils after rupture. Further, the collagen type III expression at week 3 in the treated group has brown fibers which created continuity in the bone tendon interface (Figure 7). At 6 weeks, the collagen type III fibers appeared denser. An increased density indicates more fibers in a given area which means increased strength and healing of the ligament. The biomechanical analysis tested the ultimate tensile strength of the tendon graft tunnel. The test found that the mean value of ultimate tensile strength was significantly higher in the treated group than in the control group at

3 and 6 weeks. Further, at 3 and 6 weeks, the treated knee had on average 36% and 15% greater ultimate tensile strength than the controls. The study also found that the treated groups at both 3 and 6 weeks had a higher reduction in tunnel diameter and interface diameter than the control and higher vascularization using MRI imaging. This indicates that the ligament has a strong anchorage site due to the decrease in tunnel diameter, and greater access to blood flow and nutrients due to the treatment of VEGF. The researchers concluded that intratunnel BM-MSCs and VEGF after ACL reconstruction enhance graft tunnel healing. This is due to the femoral tunnel treated with BM-MSCs and VEGF having more advanced healing with greater collagen type III fibers, and biomechanical strength.

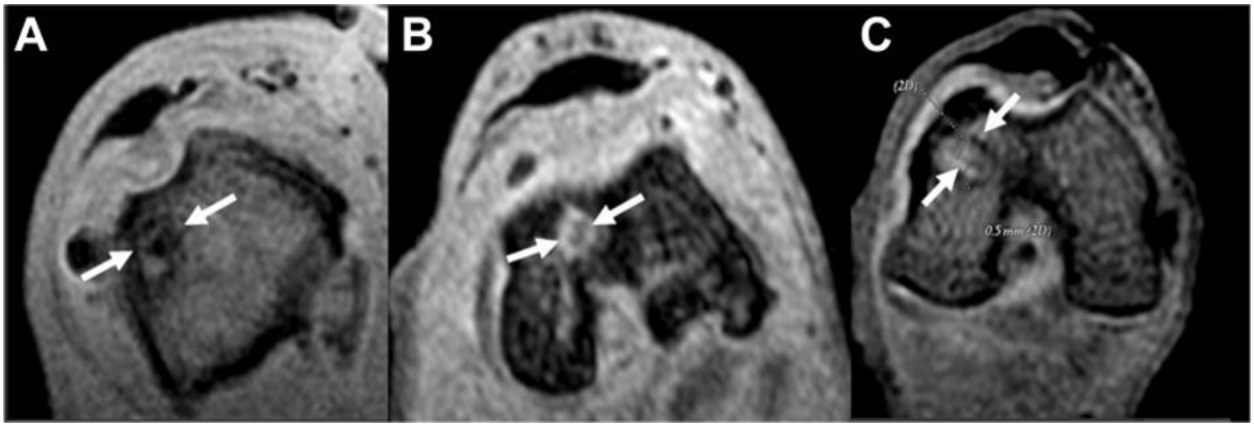

Figure 7: Axial MRI images of the femoral tunnel. (A) Specimen from control group 3 weeks. (B) Specimen from treated group 3 weeks. (C) Specimen from treated group week 6 (Morimoto et al. (2021)).

In a study done by Chen et al. (2012), the use of sodium hyaluronate as a drug-release system for VEGF was tested to see if it improves graft revascularization in anterior cruciate ligament reconstruction in rabbits. Due to the short half-life of VEGF, sodium hyaluronate (SH) was added as a delivery system to increase the time VEGF was available for the ligament. This is because, in previous studies, SH has been proven to be a carrier method for drug delivery that delays the drug release rate. Due to this delay in release rate, VEGF can be available for a longer amount of time in the place of application. In the reconstruction, the bone-patellar

tendon-bone (B-PT-B) ligament was soaked in the VEGF/SH formulation and implanted in a rabbit model to regenerate the ACL. The study hypothesized that using VEGF mixed with SH enhances the angiogenesis of a graft by prolonging the action time of VEGF. There were five groups in the study. The first group (A) B-PT-B allografts were soaked in VEGF and SH and were transplanted into the knee joints. The rest of the groups were controls. Six limbs were harvested from each group at 2, 4, and 8 weeks after surgery. The study then performed biomechanical analysis, and immunohistological evaluations for VEGF, and CD31, which is a marker for vascular endothelial cells. The biomechanical analysis revealed that the ultimate failure load of the allograft soaked in VEGF solution was significantly lower than that of the allograft soaked in SH or PBS solution 2 weeks after ACL reconstruction. Meanwhile, the ultimate failure load of groups with VEGF became significantly higher than that of the other groups at 4 and 8 weeks. Ultimate failure load is a measure of weight that can be applied to the ligament before the ligament ruptures. This determined that the biomechanical characteristics of the graft decreased at an early phase and then increased apparently later by using exogenous VEGF. Further histological and immunological results indicated that at 2 weeks, the grafts in rabbits treated with VEGF (group A) were filled by a few vessel lumina. This is important because it indicates that there is little vessel formation, indicating less vascularization and blood flow to the area. No vascular endothelial cells were observed at 2 weeks in the control groups. Further, group A had more CD31 positive cells compared to controls lacking these factors. The data for microvessel density revealed that more significant vascularization occurred in group A than in the other groups at every time point. Over time, the microvessel density increased gradually in the control groups, but not more than the treated groups. The increase in microvessel density indicates greater blood flow to the area. Greater blood flow is indicative of increased vascularization, which can increase healing due to the availability of nutrients brought by blood flow. The study concluded that VEGF enhances the revascularization of allografts after ACL reconstruction and that the application of SH to VEGF was useful by acting as a drug-

release system of VEGF, leading to an acceleration in the process of graft remodeling (Chen 2012).

A study by Yoshikawa et al. (2009) tested whether the local administration of VEGF on a tendon grafted to reconstruct the ACL of a sheep would enhance angiogenesis and cellular infiltration in the tendon grafted to reconstruct the ACL. Further, the study tested whether local administration of VEGF could accelerate mechanical deterioration in the tendon grafted to reconstruct the ACL. Female sheep underwent ACL reconstruction using the semitendinosus tendon. Two groups were created, one in which the semitendinosus tendon was soaked in VEGF, and the other was soaked in phosphate buffer. The graft tendon was placed and left to heal over a period of twelve weeks. After twelve weeks, the tendon was removed, and histological examination and biomechanical evaluation were completed. For histological examination paraffin sections were immunostained with a monoclonal antibody against actin, which is a marker for blood vessels. In the biomechanical evaluation, the femur-graft tibia complex underwent tensile testing, along with flexion angle to determine stiffness. The study found that in the tendon treated with VEGF, the number of blood vessels and the number of cells was greater than in the group treated with PBS. This indicates that VEGF increases vascularization and blood flow in ACL repair grafts. In the biomechanical analysis, it was found that the stiffness of the treated tendon was less than that of the non-treated tendon. This indicates that VEGF can cause laxity, which can lead to instability in the knee joint.
Further, the study found the average ultimate failure load was lower in the treated group than in the non-treated group but found it to be statistically insignificant. The study demonstrated that the local administration of VEGF significantly enhances the reduction in the stiffness of the grafted tendon, although the effect of VEGF on the reduction of the ultimate failure load was not significant. Further, through the histological analysis, exogenous VEGF accelerates angiogenesis and cellular infiltration in the tendon grafted in ACL reconstruction (Yoshikawa 2009).

In a study done by Zhang et al. (2003), the effect of exogenous VEGF on tendon healing and the regulation of other growth factors on rat Achilles tendons was tested. The tendon was ruptured, and VEGF was injected into the tunnel where the tendon was repaired after rupture. The tendon was repaired in two ways: The first way was repairing the Achilles tendon and removing it with the plantaris tendon, and the second way was to repair the Achilles tendon and keep the plantaris tendon intact (Figure 8) The control group received saline and the experimental group received VEGF (4 groups total: 2 different repair methods, 2 treatment methods). The tendons were evaluated at 1, 2, and 4 weeks for tension tensile strength and gene expression. For gene expression, the genes for factor-B, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) were looked at due to their role in proliferative responses. These growth factors cause inflammation, which can allow for enhanced surgical repair and initiate the start of angiogenesis. The greater the proliferative response caused by these factors, the faster the healing due to increasing type III collagen expression, angiogenesis, and vascular permeability. The study found that at week 1 postoperatively, when plantaris tendon was preserved, the tensile strength was significantly higher than in the control. It was then found that there was no difference in tensile strength between the two groups without the plantaris tendon support at week 1. In week 2, the group with VEGF treatment and plantaris tendon preservation had significantly higher tensile strength than the control and when the plantaris tendon was not preserved with both VEGF and saline. At week four after treatment, there was no significant difference in tensile strength among the groups. This indicates that exogenous VEGF can increase tensile strength in early postoperative stages, but is less effective as time increases. In gene expression testing it was found that transforming growth factor-B in the VEGF-treated tendon at day 4 was up-regulated in the early stage of tendon healing, whereas expression of platelet-derived growth factor, basic fibroblast growth factor, and insulin-like growth factor-1 was not significantly different among the groups. This indicates that VEGF could initiate the production of other growth factors in tendon

healing and improve fibroblast proliferation. By improving fibroblast proliferation, it can increase vascularization due to fibroblasts initiating angiogenesis to restore vascularization. One limitation of exogenous VEGF treatment is that it becomes less effective over time for tensile strength and gene expression due to VEGF's short half-life of 30 to 45 minutes (Zhang 2003).

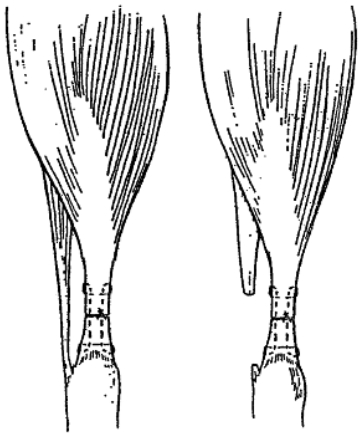

Figure 8: The Achilles tendons were repaired with the plantaris tendon removed (right) and repaired with plantaris tendon intact (left) (Zhang et al. (2003)).

Prolotherapy and Vascular Epithelial Growth Factor (VEGF)

In order to decrease the amount of healing time, doctors have begun to prescribe Nonsteroidal Anti-Inflammatory Drugs (NSAIDs). These drugs include corticosteroids, Ketorolac, and Diclofenac (Journal of Prolotherapy, 2022). The problem with these drugs, when they are injected, is that while they decrease inflammation and pain of ligament and tendon injuries, they inhibit histological, biochemical, and biomechanical properties of healing. For example, in ligaments, corticosteroid injections inhibit the fibroblasts from performing collagen synthesis. Due to this, the tensile strength decreases, causing an increase in ligament failure or retear, meaning that corticosteroid injections are now being cautioned against specifically for use in athletes (Journal of Prolotherapy, 2022).

These findings have caused doctors to lean more towards prescribing prolotherapy. This is when a combination of hypertonic dextrose, sodium morrhuate, and platelet-rich plasma is injected into the tendon or ligament after surgery or after the injury. When this is done, it increases fibroblastic proliferation, meaning that more growth factors are produced. When this was used in animal research, it was found that this therapy increased the overall tendon and ligament mass and extracellular matrix (Journal of Prolotherapy 2022). This injection is prepared by first drawing blood from the patient. This way, the body will not reject the plasma since it is from the patient's body. Once the blood is taken, the plasma and blood cells are separated, and the plasma is prepared for the injection. In some cases, growth factors that are prepared in the lab are also added. One of these factors includes VEGF. When VEGF is inserted, it promotes angiogenesis and decreases healing time due to the increase of vascularization (Rabago et al., 2009). Along with VEGF, stem cells can also be added as they can help aid in the absorption of the plasma along with other growth factors.

The injection itself can be done in three ways. The first way is if the tendon or ligament is found in a joint. Due to joints like the knee or elbow having synovial fluid around the connective tissue, the injection can be placed into the synovial fluid (Rabago et al., 2009). This allows for absorption into the entire membrane of the tendon/ligament. The next method is endogenous injection. This is when the components of the injection are placed inside the injured tendon or ligament. This method is applied right after surgery once the ligament or tendon is repaired. The final method is when the components are injected into the extracellular membrane of the tendon or ligament. Unlike the endogenous method, the components are placed just around the tendon or ligament (Beazley-Long et al., 2013). In a review by Rabago et al. (2009) shown that patients that received prolotherapy treatment reported a lower pain score that was statistically significant ($p<0.05$). This indicates that prolotherapy treatment is beneficial for patient’s pain management. Further, biomechanical elbow function assessment (polidocanol and prolotherapy) 3 weeks after injection has been shown to increase range of motion (Beazley-Long et al., 2013). The findings

of both of these reviews indicates increased vascularity on ultrasound (autologous whole blood and polidocanol). Therefore, prolotherapy is a beneficial treatment due to decreasing patient pain and increasing range or motion and vascularization.

Long-Term Results of VEGF versus Traditional Reconstruction:

Anterior cruciate ligament (ACL) injuries are common in individuals who participate in jumping and pivoting sports. When an injury to this ligament happens, most undergo surgical reconstruction to enable them to return to pre-injury activity. It has been found that normal ACL reconstruction (ACLR) can lead to an increase in osteoarthritis (Sepúlveda et al. 2017). Osteoarthritis is the degeneration of joint cartilage and the underlying bone that causes pain and stiffness (Sepúlveda et al. 2017). After surgery, rehabilitation is needed with intense physical therapy. After surgery, it has been found that neuromuscular deficits in athletes can persist for up to 9 to 12 months after the procedure (Sepúlveda et al. 2017).

Ligamental damage even with surgical reconstruction can lead to long-term effects, including osteoarthritis, loss of motion or stiffness, and pain. Pain is a common limiting factor that may hinder a patient's return to sports after ACL reconstruction (Sepúlveda et al. 2017). Due to pain being common in preventing the return to sport, treatments using platelet-rich plasma and VEGF may be used to achieve faster healing. This helps with pain due to increased vascularization to the area, which increases oxygen to the area of injury, decreasing healing time and decreasing the time of symptom duration like pain. Even with treatment, re-ruptures can occur. It was found that those who do return to sports after reconstruction have between 4% and 27% chance of re-ruptures (Sepúlveda et al. 2017).

In human studies, VEGF injections have been linked to shorter symptom duration. In a study by Scott et al. in 2008, 32 patients with patellar tendinitis were injected with VEGF after standard surgical tendon repair to see the effects. Before the VEGF treatment, the patients had severe enough pain to prevent them from participating in activities at the preinjury level. The

study found that the patients who received VEGF treatment in the patellar tendon had a shorter symptom duration of pain (12 ± 7.8 months) than patients with no VEGF treatment (32.8 ± 23.5 months). Tendonitis following surgical reconstruction is common due to joint stiffness, loss of motion, and scar tissue formation. Under normal circumstances, it can take 12 to 24 months for tendonitis symptoms to dissipate or improve to a state that allows presurgical activity. The study by Scott et al. indicates that VEGF can reduce the symptom duration of tendonitis after surgical repair, and due to a decrease in symptom duration, can get patients back to preinjury activity.

Further human studies have shown that VEGF can decrease the time of healing. In the case study previously mentioned by Morimoto et al. 2021, a 21-year-old basketball player with an Achilles tendon rupture was treated with freeze-dried platelet-derived factor VEGF at 4 weeks postoperatively. The study found that the patient could return to play at the pre-injury level without any symptoms and mechanical dysfunctions at 3 months after surgery (Morimoto et al. 2021). A normal Achilles tendon rupture can take from 6 to 12 months to heal and allow preinjury activity. The study followed the patient for two years and found that postoperatively, the patient could play basketball without symptoms including pain, and had not had a re-rupture. Therefore, the use of VEGF in ruptured tendons and ligaments may shorten symptom duration and shorten healing time.

Further, in a study done by Owens et al. (2011) platelet rich plasma (PRP) was injected into ten patients with chronic mid-substance Achilles tendinopathy. The patients were injected with PRP, which contained VEGF and platelet derived growth factor (PDGF). The patients received the injection into the tendon and MRI imaging and questionnaires were done between 7 and 24 months. The study found that clinically all the patients except one reported improvement in their pain symptoms. Since all but one obtained enough relief from injection to avoid open surgery, the injection of PRP seemed to provide substantial pain relief to patients with midsubstance Achilles tendinopathy. Due to this, PRP with VEGF helped decrease the pain from those suffering from midsubstance Achilles tendinopathy (Owens et al. 2011).

Previous studies have shown that VEGF increases vascularization by stimulating angiogenesis. Angiogenesis is the formation of new networks of blood vessels, which creates blood flow to the area where blood vessels are created. With the introduction of blood flow caused in part by VEGF release, oxygen and nutrients can be delivered to that area. The studies done by Zang et al. (2003), Yoshikawa et al. (2009), Chen et al. (2012), and Fan et al. (2016), found an increase in fibroblast proliferation or increased lumina which is indicative of blood vessel formation. With increased blood vessel formation there is more access to nutrients and oxygen, which creates the proper conditions for healing. By increasing vascularization with VEGF and creating the optimum environment for healing, the time it takes to heal may decrease when compared to treatments without VEGF. This is because VEGF promotes vascularization and the conditions for healing, which will ultimately lead to faster healing.

Conclusion:

The use of VEGF endogenously and exogenously in tendon and ligament repair is beneficial as it decreases the duration of symptoms and decreases healing time. Tendons and ligaments are essential tissues needed to move the body; without them functioning properly, movement is impaired, and the quality of life is decreased for many individuals. Surgery is often needed to fix ruptures but does not always guarantee full recovery and the chances of re-injuring that tendon or ligament increase. This has led researchers to turn to VEGF. The studies in this review have shown that VEGF increases tensile strength, decreases inflammation, and increases vascularization. In studies using exogenous VEGF, it was found that VEGF increases collagen type III fibers, which increases biomechanical strength, and improves graft revascularization. From the research, it can be concluded that endogenous injection of VEGF is more beneficial than exogenous due to endogenous not causing laxity of the connective tissue.

Table 1: Overall summary of findings of endogenous and exogenous treatments of VEGF

	Endogenous VEGF	**Exogenous VEGF**
Tensile Strength	Increase	Cause laxity of the connective tissue (Chen)
Collagen III	Increase	Increase
Collagen I	Increase	No change
Vascularization	Increase	Increase

VEGF is beneficial when compared to normal post-operative results due to it decreasing the duration of symptoms and decreasing healing time. Using VEGF injection treatments, patients can return to pre-injury activity at a higher rate than those not using VEGF treatment. Together, these studies show that the use of VEGF in tendon and ligament healing should be further utilized in future therapies. In my opinion I would recommend the use of surgical reconstruction with physical therapy until VEGF treatment is approved by the Food and Drug Administration (FDA). The animal studies reviews show promise in decreasing healing time, along with increasing vascularization and tensile strength. Until the treatment is approved in humans, surgical intervention and physical therapy is recommended. Further, I would not recommend the use of corticosteroid injections to reduce pain. This is a result of the injections preventing fibroblastic preliberation which hinders the healing process.

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